

## Response to *Mycobacterium bovis* BCG Vaccination in Protein- and Zinc-Deficient Guinea Pigs

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Groups of specific pathogen-free Hartley strain guinea pigs were vaccinated with viable *Mycobacterium bovis* BCG and maintained on isocaloric purified diets containing either 30 or 10% protein (ovalbumin) combined with either 50 ppm ( $\mu\text{g/g}$ ) or no added zinc. Seven weeks later the guinea pigs were skin tested with purified protein derivative and killed. Both protein and zinc deficiencies had a significant negative impact on growth of the guinea pigs. Both groups consuming the 10% protein diet also demonstrated significant reductions in hematocrit, total serum proteins, and serum albumin, as well as diminished spleen weight. Plasma zinc concentrations were reduced in both low-zinc groups to less than half the value observed in control guinea pigs. Protein deficiency, alone or combined with zinc deficiency, resulted in increased tissue levels of viable *M. bovis* BCG in the inguinal lymph nodes and subcutaneous vaccination nodule. These same groups exhibited significant impairment in the ability to mount a delayed hypersensitivity reaction. Phytohemagglutinin-driven polyclonal T cell blastogenesis in vitro was significantly diminished in the peripheral lymphocytes of both protein- and protein-zinc-deficient animals at low mitogen doses, but only in the protein-zinc-deficient guinea pigs as the dose of phytohemagglutinin was increased. These results suggest that dietary protein and zinc deficiencies, alone or combined, interfere with immunological responses of the host to vaccination with *M. bovis* BCG.

Protein-calorie malnutrition in humans has been associated repeatedly with perturbations in immunological function and alterations in disease resistance (9, 31). The role of specific nutrients or combinations of nutrients has been difficult to evaluate because of the complexity and diversity of the malnourished state in humans. However, evidence from such studies, along with data generated in experimental animal models, suggests that single-nutrient deficiencies are often associated with the same types of immune impairment seen in protein-calorie malnutrition (4, 29). Some investigators have suggested that deficiencies of essential micronutrients such as zinc may explain, in large measure, the immunological abnormalities seen in protein-calorie malnutrition (13).

Zinc deficiency has been associated clearly with impaired humoral and cell-mediated immunity, both in humans with congenital (24) or acquired deficiencies (2, 26) and in rodents made deficient by dietary manipulation (3, 12). Zinc is required for the function of a large number of metalloenzymes, including some involved in

DNA and RNA synthesis (27). Zinc in certain concentrations may act as a mitogen for lymphocytes in vitro (28) and appears to be required for the proliferative event (10). A direct effect of zinc nutriture on thymus size has been demonstrated in both humans (14) and rodents (3, 8, 12).

The relevance of zinc-related immune dysfunctions for disease resistance in the deficient host has not been adequately examined. Compared with numerous studies of antibody production and mitogen-driven lymphoproliferation in zinc-deficient mice or rats (3, 8, 12) only a few have tested the response to infectious challenge (25), and there is little published evidence of such studies in guinea pigs (17). In previous experiments with this model, we have documented the impact of protein-calorie malnutrition on several aspects of cell-mediated immunity and acquired cellular resistance in guinea pigs vaccinated with viable *Mycobacterium bovis* BCG (20, 21).

In the present study, we compared the response to *M. bovis* BCG in groups of guinea pigs maintained on diets that were deficient in either zinc alone, protein alone, or zinc and protein, as compared with animals fed a fully-supplemented

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TABLE 1. Composition of the experimental diets

Ingredient	Diet composition (g/kg of diet)			
	I (high protein, high zinc)	II (high protein, low zinc)	III (low protein, high zinc)	IV (low protein, low zinc)
Egg white albumin	300	300	100	100
Sucrose	350	350	350	350
Cornstarch	22	22	222	222
Nonnutritive fiber	180	180	180	180
Mineral mix <sup>a</sup>	40 <sup>b</sup>	40	40 <sup>b</sup>	40
Potassium acetate	27	27	27	27
Magnesium oxide	5	5	5	5
Vitamin mix <sup>c</sup>	12	12	12	12
Ascorbic acid	2	2	2	2
Choline bitartrate	2	2	2	2
Corn oil	60	60	60	60

<sup>a</sup> Provided the following minerals at the designated levels (g/kg of diet): CaCO<sub>3</sub>, 10.0; CaHPO<sub>4</sub> · 2H<sub>2</sub>O, 25.0; MgSO<sub>4</sub>, 0.5; MgCO<sub>3</sub>, 1.0; NaCl, 2.8; Fe<sub>6</sub>H<sub>5</sub>O<sub>7</sub> · 5H<sub>2</sub>O, 0.6; KIO<sub>3</sub>, 0.038; MnSO<sub>4</sub> · H<sub>2</sub>O, 0.8; CuSO<sub>4</sub>, 0.036; CoCl<sub>2</sub> · 6H<sub>2</sub>O, 0.03; AlK(SO<sub>4</sub>)<sub>2</sub> · 12H<sub>2</sub>O, 0.007; NaF, 0.04; KCl, 4.5; CrK(SO<sub>4</sub>)<sub>2</sub> · 12H<sub>2</sub>O, 0.0048; Na<sub>2</sub>SeO<sub>3</sub> · 5H<sub>2</sub>O, 0.00035.

<sup>b</sup> ZnCO<sub>3</sub> was added to mineral mix to provide 50 ppm zinc.

<sup>c</sup> American Institute of Nutrition (AIN) vitamin mix was modified by the addition of the following (g/kg of mix): calcium pantothenate, 1.6; folic acid, 1.0; retinyl palmitate, 3.2 (500,000 IU/g); *dl*- $\alpha$ -tocopherol, 10.0 (250 IU/g); biotin, 0.38.

diet. The host responses that we measured included delayed hypersensitivity, mitogen-driven lymphocyte blastogenesis *in vitro*, and the ability to control mycobacterial populations at the site of injection.

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## MATERIALS AND METHODS

**Experimental animals.** Outbred, albino, specific pathogen-free female guinea pigs, weighing 250 to 350 g, were obtained from a commercial source (Hartley-COBS, Crl:(HA)BR; Charles River Breeding Laboratories, Inc., Wilmington, Mass.). They were housed individually in polycarbonate cages on stainless steel mesh floors and provided food in stainless steel feeders and demineralized water (essentially zinc-free) *ad libitum*. Each animal was randomly assigned to an experimental diet treatment group. Body weights were recorded weekly during the experiment.

**Experimental diets.** The experimental diets, based upon ovalbumin as the protein source, were designed to meet current recommended nutritional requirements for guinea pigs (23). Four diets (I, II, III, and IV) representing factorial combinations of two levels of protein (30 and 10%) and two levels of added zinc (50 ppm [ $\mu$ g/g] and 0 ppm) were used. The diets were isocaloric, with the proportion of corn starch and ovalbumin varying inversely to provide the desired protein content. The formulation of the four diets is presented in Table 1. The food was given as a powder, and fresh diet was provided every other day.

***M. bovis* BCG vaccination.** On the same day the experimental diets were started, the guinea pigs were vaccinated with viable *M. bovis* BCG vaccine (Copenhagen 1331, Statens Seruminstitut, Copenhagen, Denmark). Each animal received 0.1 ml of saline solution

containing approximately 10<sup>3</sup> viable bacilli subcutaneously over the left inguinal region. The viability of the vaccine was determined by plating appropriate dilutions on oleic acid albumin agar (Difco Laboratories, Detroit, Mich.).

**PPD skin tests.** Two days before sacrifice, guinea pigs received two intradermal injections of 0.1 ml each, containing either 5 or 100 tuberculin units (TU) of purified protein derivative (PPD; PPD-RT23 from Statens Seruminstitut), on a shaved area of the side. The reactions were measured with a transparent plastic ruler 48 h later, and the mean diameter of induration in millimeters was recorded.

**Autopsy procedure.** Seven weeks after initiation of the experimental diets and vaccination, groups of six to eight guinea pigs from each treatment were killed by cervical dislocation. A 5- to 7-ml blood sample was taken immediately by cardiac puncture into a 10-ml syringe containing sufficient preservative-free heparin (Sigma Chemical Co., St. Louis, Mo.) to provide 50 U/ml of blood. The abdominal cavity was opened aseptically, and the inguinal lymph nodes, subcutaneous vaccination nodule, and spleen were removed to separate sterile petri dishes and weighed. The lymph nodes and vaccination nodule were then homogenized in 2 ml of sterile 2% albumin solution in a Teflon-glass homogenizer. Appropriate dilutions were inoculated onto duplicate oleic acid albumin agar plates that were incubated at 37°C for 3 to 4 weeks. The number of *M. bovis* BCG colonies were counted and the results expressed as mean log<sub>10</sub> viable *M. bovis* BCG per milligram (wet weight) of tissue.

Hematocrits were determined on the blood samples by using a standard clinical procedure. Serum total proteins were quantified by the Lowry method (18), and the serum albumin concentrations were calculated by quantitative protein electrophoresis on cellulose acetate strips. Plasma zinc concentrations were determined with an atomic absorption spectrophotometer

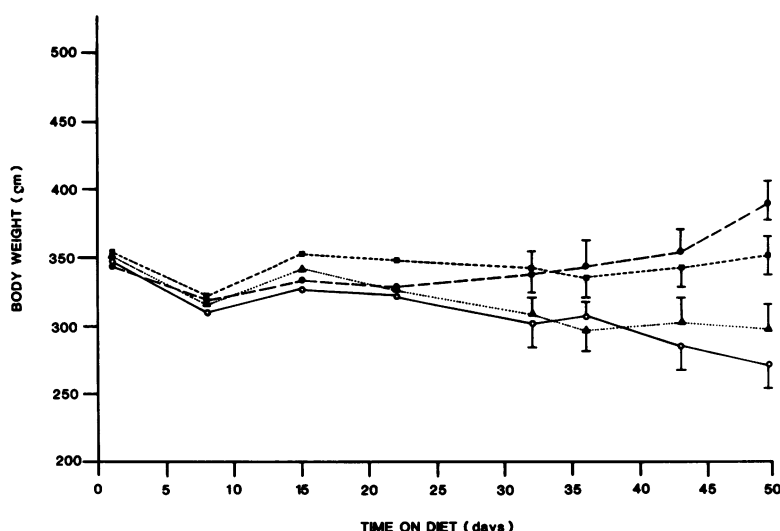


FIG. 1. Growth of *M. bovis* BCG-vaccinated guinea pigs maintained on purified diets containing high protein and high zinc (●), high protein and low zinc (■), low protein and high zinc (▲), or low protein and low zinc (○). Vertical bars indicate standard error of the mean of eight animals.

(model 303; The Perkin-Elmer Corp., Norwalk, Conn.).

**Lymphocyte blastogenesis.** A whole blood culture technique was used exactly as previously described (20). Samples (250  $\mu$ l) of a 1:6 dilution of heparinized blood in tissue culture medium (RPMI 1640; Microbiological Associates, Bethesda, Md.) plus 10% fetal bovine serum were placed into the wells of a microtiter plate (Microtest II; Falcon Plastics, Oxnard, Calif.). Triplicate cultures were stimulated with four dilutions of phytohemagglutinin (PHA; PHA-P from Difco) representing relative concentrations of 1, 2, 10, and 20  $\mu$ l per well. After 72 h of culture at 37°C in 5% CO<sub>2</sub> atmosphere (including a final 24-h pulse with 0.8  $\mu$ Ci of tritiated thymidine per well), the cultures were harvested onto fiberglass filter disks and counted in a liquid scintillation counter (LS 8000; Beckman Instruments, Inc., Fullerton, Calif.). Counts were corrected for background and quenching and adjusted to represent the response of 10<sup>6</sup> lymphocytes. The data are presented as a blastogenic index, which is defined as the ratio of counts per minute in mitogen-stimulated cultures to the counts per minute in nonstimulated cultures of the same animal's cells.

**Statistical analysis.** The analysis of variance was used to test for the effect of each dietary treatment (i.e., protein and zinc) on the dependent variables measured. When appropriate, Student's *t* test was used to assess the significance of differences between group means. The comparisons made were specified by the analysis of variance results for each dependent variable. If the protein term alone was significant, the appropriate comparisons were adequate versus low protein within each zinc level (i.e., group I versus group III, group II versus group IV). If the zinc term alone was significant in the analysis of variance, the appropriate comparisons were adequate versus low zinc within each protein level (i.e., group I versus group II, group III versus group IV). All appropriate

comparisons were made for each dependent variable, and only those for which significance was obtained ( $P < 0.05$ ) are indicated on the figures.

## RESULTS

**Effect of diets on indicators of nutritional status.** Figure 1 illustrates the effect of the four experimental diets on body weight. The mean weights of the treatment groups were essentially identical at the initiation of the diets and simultaneous vaccination. All guinea pigs apparently went through an adaptation phase during which food consumption was reduced and body weight dropped slightly. The first significant effect of diet on growth was observed at 32 days when the two low-protein groups were found to have lost weight compared with the two high-protein groups. This significant difference was maintained until the end of the experiment. In addition, the effect of zinc deficiency on growth appeared at 49 days, with both low-zinc groups lighter than their respective zinc-adequate controls at the same protein level.

The impact of dietary protein and zinc deficiencies on hematocrit, total serum proteins, and serum albumin is illustrated in Fig. 2. Significant reductions in all three parameters were observed in both of the 10% protein groups as compared with the fully supplemented control. Group III was significantly lower than group I, and group IV was significantly lower than group II. Zinc deficiency alone did not exert a statistically significant influence on any of these indicators in the 30% protein group. Guinea pigs maintained on a diet deficient in both protein

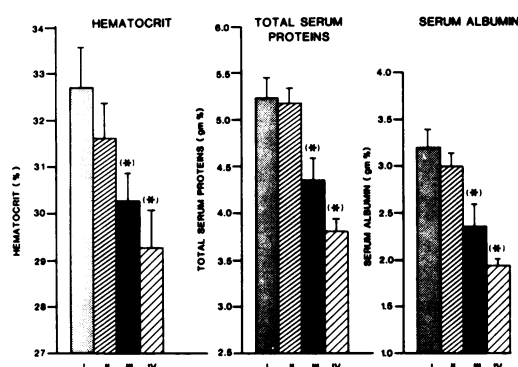


FIG. 2. Effect of diet on serum proteins and hematocrit in *M. bovis* BCG-vaccinated guinea pigs maintained on purified diets containing high protein and high zinc (I), high protein and low zinc (II), low protein and high zinc (III), or low protein and low zinc (IV). Vertical bars indicate standard error of the mean; an asterisk indicates significant difference from the corresponding control ( $P < 0.05$ ).

and zinc (group IV) had significantly lower concentrations of total proteins and albumin than their zinc-adequate, low-protein counterparts (group III).

Zinc status, as measured by plasma concentrations of elemental zinc, was significantly influenced by dietary treatment. Compared with the high-protein-high-zinc group ( $0.880 \pm 0.048$   $\mu\text{g/ml}$ ), plasma zinc levels were significantly diminished in both the high-protein-low-zinc ( $0.433 \pm 0.065$   $\mu\text{g/ml}$ ) and low-protein-low-zinc ( $0.368 \pm 0.078$   $\mu\text{g/ml}$ ) groups. The guinea pigs fed the low-protein-high-zinc diet had somewhat lower plasma zinc concentrations, which were not significantly different from control guinea pigs ( $0.747 \pm 0.090$   $\mu\text{g/ml}$ ).

Both low-protein diets had a marked influence on lymphoid tissue development as indicated by significant reductions in both absolute and relative spleen weight (Fig. 3). Animals in group III demonstrated significantly lower spleen weights than animals in group I, and the values for group IV were significantly lower than those for group II. Zinc deficiency alone had no measurable effect on spleen size at either dietary protein level.

**Extent of *M. bovis* BCG infection.** Figure 4 illustrates the effect of dietary protein and zinc on the number of viable *M. bovis* BCG recovered from the inguinal lymph nodes and subcutaneous vaccine site 7 weeks after vaccination. Both of the groups consuming the 10% protein diet had significantly more *M. bovis* BCG in the subcutaneous vaccination nodule. Groups I and III were significantly different, as were groups II and IV. There were no differences detected between low-zinc and high-zinc groups, irre-

spective of protein level in terms of the numbers of organisms in the vaccination site. In contrast, both low-zinc groups had more *M. bovis* BCG in their inguinal lymph nodes than did their respective high-zinc counterparts. Significant differences were observed between group I and group II and between group III and group IV.

**Response to PPD skin test.** Guinea pigs maintained on the 10% protein diet, irrespective of zinc level, were significantly impaired in their ability to mount a delayed hypersensitivity response to PPD (Fig. 5). The mean diameter of induration in those groups (III and IV) was reduced to less than half that observed in the fully supplemented control groups (I and II, respectively) at both the 5 and 100 TU doses of PPD. All guinea pigs responded more vigorously to the 100 TU dose, but the reactions were still impaired in both groups consuming the 10% protein diet. Although zinc deficiency alone resulted in somewhat diminished responses to 100 TU of PPD, the analysis of variance did not reveal a statistically significant zinc term.

**Mitogen-drive lymphocyte blastogenesis.** Figure 6 illustrates the blastogenic response of peripheral blood lymphocytes from *M. bovis* BCG-vaccinated guinea pigs maintained on the four experimental diets. Four graded doses of the T lymphocyte mitogen, PHA, were used, and the group mean blastogenic index was plotted at each dose. The mean counts per minute in the unstimulated (control) cultures for each of the treatment groups was: group I,  $426 \pm 123$ ; group II,  $386 \pm 134$ ; group III,  $463 \pm 89$ ; group IV,  $344 \pm 60$ . Lymphocytes from both low-

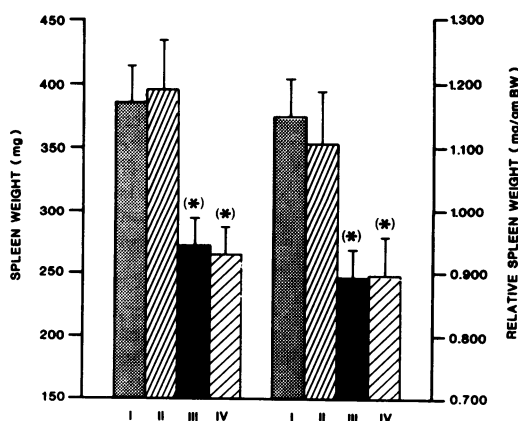


FIG. 3. Absolute and relative spleen weights of *M. bovis* BCG-vaccinated guinea pigs maintained on purified diets containing high protein and high zinc (I), high protein and low zinc (II), low protein and high zinc (III), or low protein and low zinc (IV). Vertical bars indicate standard error of the mean; an asterisk indicates significant difference from the corresponding control ( $P < 0.05$ ).



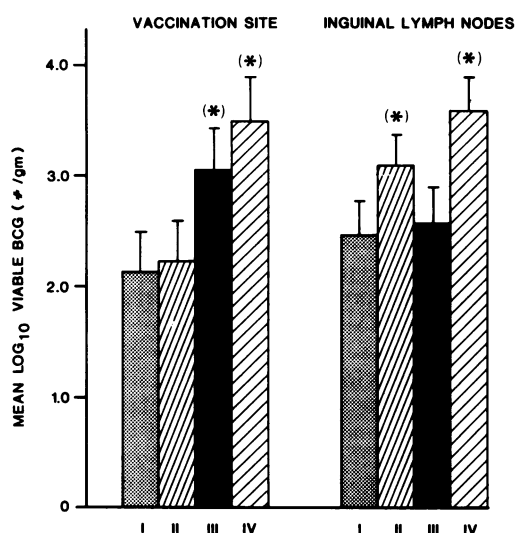


FIG. 4. Effect of diet on the number of viable *M. bovis* BCG recovered 7 weeks postvaccination from the tissues of guinea pigs maintained on purified diets containing high protein and high zinc (I), high protein and low zinc (II), low protein and high zinc (III), or low protein and low zinc (IV). Vertical bars indicate standard error of the mean; an asterisk indicates significant difference from the corresponding control ( $P < 0.05$ ).

protein groups exhibited impaired blastogenesis at the two lowest doses of PHA. Only lymphocytes from the protein- and zinc-deficient group, however, continued to respond subnormally as the dose of PHA in the cultures was increased. At the highest dose of PHA used, the blastogenic activity of cells from the low-protein-high-zinc group was not different from that of the two high-protein groups, whereas the response of lymphocytes from the group deficient in both protein and zinc was still significantly diminished.

### DISCUSSION

Body weight and serum biochemical indicators confirmed that guinea pigs consuming diets deficient in zinc, protein, or both for 7 weeks were measurably malnourished with respect to the limiting dietary nutrients. Evidence for protein deficiency included significant weight loss between 3 and 7 weeks, significant reductions in serum total proteins and albumin, and reduced relative spleen weight. The zinc deficiency was moderate, with growth retardation not appearing until 7 weeks. Significant reductions in plasma zinc concentrations reflected low dietary intakes. The effect of the 10% ovalbumin diet on growth and protein status in this experiment was very similar to that reported earlier for 7.5 and 10% casein diets in guinea pigs (20, 21). The plasma zinc levels in fully supplemented guinea

pigs ( $0.880 \pm 0.048 \mu\text{g/ml}$ ) were somewhat lower than those observed previously in normal guinea pigs. Alberts et al. reported plasma concentrations of nearly  $2.00 \mu\text{g/ml}$  in chow-fed guinea pigs (1), whereas McBean et al. found levels of  $1.36 \mu\text{g/ml}$  in their guinea pigs consuming normal amounts of zinc (19). The plasma zinc concentrations in both zinc-deficient groups were very similar to those reported by McBean et al. (19) and somewhat lower than those observed by Alberts feeding a diet containing less than 3 ppm of zinc (1). These differences are probably due to the fact that our guinea pigs were infected with *M. bovis* BCG. Infection with mycobacteria is known to alter plasma zinc levels due to redistribution from the circulation to the liver (7).

The protein and zinc deficiencies, either singly or in combination, significantly altered the response of guinea pigs to vaccination with *M. bovis* BCG, but the nature and magnitude of the impairment varied with test parameters and test conditions. For example, acquired resistance, as evidenced by failure to mount a normal delayed hypersensitivity response to PPD, was impaired in protein-deficient animals (groups III and IV) at both the 5- and 100-TU doses, regardless of zinc status (Fig. 5). When the 100-TU dose was used, there was a trend for zinc deficiency alone to have a deleterious effect (group II), although the difference was not statistically significant. With the 100-TU dose, the combined protein and

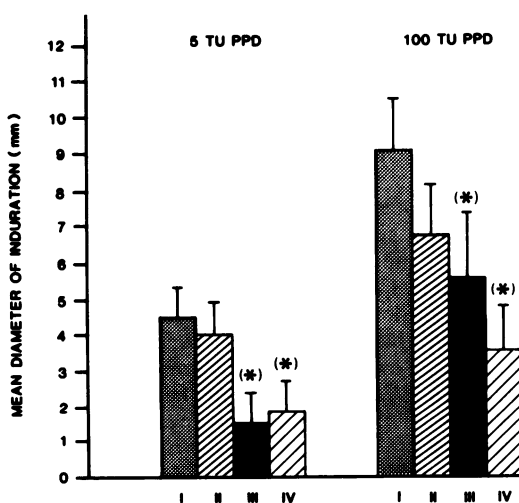


FIG. 5. Delayed hypersensitivity reactions to two doses of PPD in *M. bovis* BCG-vaccinated guinea pigs maintained on purified diets containing high protein and high zinc (I), high protein and low zinc (II), low protein and high zinc (III), or low protein and low zinc (IV). Vertical bars indicate standard error of the mean; an asterisk indicates significant difference from the corresponding control ( $P < 0.05$ ).

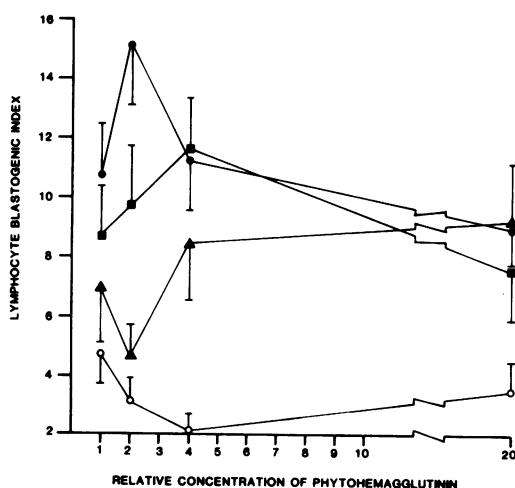


FIG. 6. Blastogenic dose response to PHA of lymphocytes from *M. bovis* BCG-vaccinated guinea pigs maintained on purified diets containing high protein and high zinc (●); high protein and low zinc (■), low protein and high zinc (▲), or low protein and low zinc (○). Vertical bars indicate standard error of the mean.

zinc deficiencies (group IV) resulted in a more severe impairment than either a simple zinc or a simple protein deficiency alone. The observed effect of protein malnutrition in producing tuberculin anergy after vaccination with *M. bovis* BCG is consistent with results reported by us and others (20, 21, 30, 32). A similar effect with zinc deficiency has been reported by Golden et al. in humans (13) and by Fraker et al. in rodents (12), using other antigens. The fact that we did not observe a statistically significant impairment with zinc deficiency alone may have been due to the relatively moderate zinc deficiency obtained in this study, as indicated by the lack of effect on growth rate until late in the experimental period. However, it is important to note that this moderate level of zinc deficiency, which alone did not result in a statistically significant impairment in ability to mount a normal delayed hypersensitivity response to PPD, did act synergistically with a protein deficiency to give a more severe impairment than either type of deficiency alone.

Further evidence for an impaired response to *M. bovis* BCG was seen in the failure of protein- and zinc-deficient guinea pigs to control mycobacterial populations at the site of vaccination or the lymph nodes draining that site (Fig. 4). We have already published results similar to those reported here in protein-malnourished guinea pigs (20, 21). Bhuyan et al. have previously documented reduced inflammatory responses and increased bacillary loads in stained sections of tissues from protein-deficient, *M. bovis* BCG-vaccinated rabbits and guinea pigs (5, 6). A significant effect of zinc nutriture alone was seen

in the lymph nodes. Pekarek et al. observed increased susceptibility to experimental tularemia in zinc-deficient rats (25).

The dose response of splenic lymphocytes to PHA in vitro reveals a dissociation between protein- and zinc-deficient guinea pigs (Fig. 6). Whereas protein deficiency, with or without concomitant zinc deficiency, was responsible for significant reductions in mitogenesis in cultures exposed to low doses of PHA, only splenocytes from protein- and zinc-deficient guinea pigs failed to recover normal responsiveness when the dose of PHA was increased 20-fold. These results indicate the importance of mitogen dose and confirm our previous observations in protein-malnourished *M. bovis* BCG-vaccinated guinea pigs (20, 21). However, our results relative to the effect of zinc deficiency on lymphocyte blastogenesis are not totally consistent with those reported by others. Zinc deficiency has been associated with reductions in PHA-driven lymphoproliferation in rats (16, 25), mice (3), and humans (26). Proliferative responses to other mitogens, including concanavalin A and pokeweed mitogen, have also been impaired by experimental zinc deficiencies in rodents (3, 16). Conversely, our results showed that a moderate zinc deficiency resulted in an impaired blastogenesis only when it occurred concomitantly with a protein deficiency. Animals on diets II (high protein, low zinc) and IV (low protein, low zinc) had low plasma zinc concentrations as compared with animals on the other two diets. Yet only animals on diet IV showed an impairment in blastogenesis at all PHA doses. Animals on diet II, although also exhibiting low serum zinc levels, had a relatively normal blastogenic response as compared with controls. Although the recovery of responsiveness by lymphocytes from group III (low protein, high zinc) at high PHA doses suggests that those lymphocytes were not irreversibly impaired, it is also possible that this apparent recovery was actually due to the suppressive effects of high PHA doses on the normal cells of the other two groups.

Taken together, our results demonstrate that a moderate zinc deficiency superimposed on protein malnutrition has a deleterious effect on host responses to vaccination with *M. bovis* BCG. As would be expected from the profound influence of dietary zinc on the thymus (8, 14), T lymphocyte function is altered as evidenced by tuberculin anergy, failure to control mycobacterial accumulation in the tissues, and hyporesponsiveness to a T lymphocyte mitogen in vitro. The impaired ability to limit *M. bovis* BCG populations in vivo, although statistically significant, was not absolute. We have shown that protein-malnourished guinea pigs eventually reduce mycobacterial populations to control levels in the

tissues (21). Effective acquired resistance, especially against a facultative intracellular pathogen like *M. bovis* BCG, ultimately requires a population of competent macrophages. We have reported evidence for enhanced macrophage enzymatic activity in protein-deficient guinea pigs (22), and others have observed enhanced macrophage migration, antibody-dependent cellular cytotoxicity, and natural killer cell activity in zinc deficiency (8, 15, 17, 28). It is possible that other mechanisms of antimicrobial resistance may compensate in malnourished individuals for impairment of classical T lymphocyte-mediated macrophage activation. In addition, a direct effect of suboptimal concentrations of zinc on growth of *M. bovis* BCG in vitro has been reported and may influence the outcome of this altered host-parasite interaction (11).

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